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PRINCIPAL INVESTIGATOR: Vaqar M. Adhami, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, WI 53706

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14. ABSTRACT Microtubule associated proteins (MAP)-2 a component of the MAP family, is a marker for neurons and its immuno-reactivity has been demonstrated in several neoplasms. We hypothesized that MAP-2 expression is deregulated in EGFR over-expressing breast cancers thus rendering them resistant to conventional therapy. Here we show that loss of MAP-2 expression in breast cancer cells during sustained activation of the EGFR results in resistance to chemotherapeutic drugs. We observed higher expression of MAP-2 in EGFR over-expressing cells than in non-EGFR over-expressing cells both at protein and mRNA levels. MCF-7 and MCF-10A cells were challenged with increasing doses of EGF (25-150 ng/ml) and examined for the expression of phosphorylated EGFR. We observed that expression of MAP-2 in cell lines challenged with EGF increased with increasing doses of EGF, however, its expression was almost completely lost at concentrations >100 ng/ml of EGF treatment. This observation suggested a possible mechanism of resistance in breast cancer patients with EGFR over-expression. We also found increasing resistance to growth inhibition by docetaxel in cells that were challenged with higher concentrations of EGFR (>50 ng/ml). This suggested that over expression of EGFR signaling in breast cancers could, in fact, be responsible for resistance to therapeutic agents. The loss of MAP-2 expression could have implications in treatment of breast cancers over-expressing the EGFR and exhibiting resistance to conventional therapy.					
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Annual Report for Award Number W81XWH-04-1-0741

Title: Association between microtubule associated protein-2 and the EGFR signaling in breast cancer.

Summary: Epidermal growth factor receptor (EGFR)/HER1 is expressed at high levels in ~20% of breast cancers and correlates with poor disease prognosis. One mechanism that contributes to the generation and maintenance of cancerous cells is the disruption of the cellular cytoskeleton. Microtubule associated proteins (MAPs) are major components of cytoskeleton proteins associated with microtubule assembly and its stabilization. MAP-2, a component of the MAP family, is a marker for neurons and its immunoreactivity has been demonstrated in several neoplasms. We hypothesized that MAP-2 expression is deregulated in EGFR overexpressing breast cancers thus rendering them resistant to conventional therapy. Here we show that loss of MAP-2 expression in breast cancer cells during sustained activation of the EGFR results in resistance to chemotherapeutic drugs. We first evaluated the expression of MAP-2 in a range of breast cancer cells and found that all cell lines expressed varying degrees of MAP-2. However when compared on the basis of their EGFR status, we observed higher expression of MAP-2 in EGFR overexpressing cells than in non-EGFR overexpressing cells both at protein and mRNA levels. To investigate whether the expression of MAP-2 is regulated by the expression of EGFR we selected two cell lines based on their responsiveness to EGF. MCF-7 and MCF-10A cells were challenged with increasing doses of EGF (25-150 ng/ml) and examined for the expression of phosphorylated EGFR. We observed that MCF-7 cells were weakly and MCF-10A cells were strongly stimulated when challenged by increasing doses of EGF. We next examined the expression of MAP-2 in cell lines challenged with EGF and observed that although MAP-2 expression increased with increasing doses of EGF, its expression was almost completely lost at concentrations >100 ng/ml of EGF treatment. This observation suggested a possible mechanism of resistance in breast cancer patients with EGFR overexpression. Based on these observations we conclude that with constant EGF stimulation, cells lose their MAP-2 expression that is required for microtubule stabilization and subsequent cell cycle arrest. In next series of experiments we sought to understand how cells would respond to microtubule disrupting agents after they are challenged with increasing doses of EGF. We found increasing resistance to growth inhibition by docetaxel in cells that were challenged with higher concentrations of EGFR (>50 ng/ml). This suggested that over expression of EGFR signaling in breast cancers could, in fact, be responsible for resistance to therapeutic agents. The loss of MAP-2 expression could have implications in treatment of breast cancers overexpressing the EGFR and exhibiting resistance to conventional therapy.

INTRODUCTION:

The primary determinants that govern the growth and differentiation of breast cancer cells are largely unknown. One mechanism that contributes to the generation and maintenance of cancerous cells is the disruption of the cellular cytoskeleton. Microtubule associated proteins (MAPs) are major components of cytoskeleton proteins associated with microtubule assembly that are developmentally regulated and function in stabilization and *in vitro* assembly of microtubules (1). It has been suggested that MAPs function as linkers between microtubules and

cellular structures including other cytoskeleton elements. Microtubule associated protein-2 (MAP-2), a component of the MAP family, has been shown to be expressed in neuronally differentiated cells, and has been previously used as a sensitive and specific marker for neurons (2). Immunoreactivity of MAP-2 has been demonstrated in several neoplasms of skin, lung, brain and malignant melanomas, and it has been observed in some cancers that MAP-2 expression is high in tumors sensitive to microtubule disrupting agents (MDA) (3), however the expression and role of MAP-2 in breast cancer cells has not been evaluated.

While microtubule disruption remains an important therapeutic modality against breast cancer, the subsequent development of resistance and the inevitability of escape from effectiveness of MDA has been a stumbling block. Understanding the mechanism of resistance to MDA may lead to devising effective strategies against breast cancer refractory to MDA. Epidermal growth factor receptor (EGFR)/HER1 is expressed at high levels in at least 20% of breast cancers and MAP-2 also serves as a substrate for EGFR kinases (4). A possible association between MAP-2 and EGFR expression in breast cancer seems plausible.

This proposal aims at investigating the role of MAP-2 in breast cancer hitherto unknown and is designed to examine whether it has a role in drug resistance. It is also possible that high expression of MAP-2 in cancer cells may lead to microtubule stabilization and consequent withdrawal from cell cycle and subsequent induction of apoptosis. This could lead to escape from apoptosis of malignant breast cancer cells that express MAP-2. Those cells that escape this control may continue to proliferate and metastasize. Since experimental and clinical findings suggest that aberrant activation of tyrosine receptor kinases, such as HER1 pathway, play a causal role in the development of resistance in breast cancer this proposal will investigate association between MAP-2 and the EGFR pathway. Understanding how MAP-2 expression changes course in breast cancer could have therapeutic implications.

MATERIALS AND METHODS:

Cells: Normal human mammary epithelial cells were obtained from Cambrex Bioscience (Baltimore, MD). All breast cancer cells were purchased from ATCC (Manassas, VA). Cells were grown in appropriate media as recommended by the vendors. Epidermal growth factor, MTT and Docetaxel were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against MAP-2, EGFR, Erk1/2, β -Actin were purchased from Sigma (St. Louis, MO), Cell Signaling (Beverly, MA) and Upstate (Charlottesville, VA).

Immunoblot Analysis. Cells were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4 , 0.5% NP40, 1% Triton X-100, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin, pH 7.4) at 4 °C to prepare lysates. The protein concentration was determined by BCA-protein assay kit and following the manufacturer's protocol (Pierce, Rockford, IL). Appropriate amount of protein (25-50 μg) was resolved over 8-14% Tris-Glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% non-fat dry milk and probed using appropriate primary antibody in blocking buffer overnight at 4 °C. The membrane was then incubated with secondary antibody horseradish peroxidase (HRP) conjugate (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit

(Amersham Life Sciences Inc., Arlington Heights, IL). Equal loading of protein was confirmed by stripping the membrane and re-probing it with β -Actin primary antibody (Sigma, St. Louis, MO) and appropriate secondary HRP conjugate.

RNA Isolation and Reverse Transcription. Total RNA was isolated from the cells using the RNeasy Total RNA isolation kit (Qiagen, Valencia, CA) and was checked for its purity and concentration. Two μ g of total RNA was reverse transcribed using murine MLV- reverse transcriptase and oligo dT (12-18) primer from GIBCO-BRL (Life Technologies Inc., Gaithersburg, MD) for cDNA synthesis.

Semi Quantitative PCR for MAP-2. Two μ l of the RT-product was subjected to PCR in 25- μ l reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 units of Amplitaq polymerase and 40 pmoles of each primer for MAP-2 (sense primer, 5'-ATCAAATGGTCCACTAGGCG-3' anti-sense primer, 5'-GCACTTCAAGGGAAG-CTGAT-3') obtained from Oxford Biomedical Research, Inc. (Rochester Hills, MI). About 10- μ l aliquots (having equal RNA) of the reverse transcribed cDNA samples were added to the reaction mix and coamplified for 35 cycles: denaturation at 94 °C for 1 min, annealing at 53°C for 1 min, extension at 72 °C for 2 min, and final extension at 72°C for 10 min. A constitutively expressed gene, b-Actin (sense 5' ATCTGGCACCACACCTTCTACAATGAGCTGCG 3'; antisense 5' CGTCATACTCCTGCTTGCTGATCCACATCTGC 3') was used as an internal control. Ten μ l of PCR products were loaded on 1.5% agarose gels and visualized by ethidium bromide staining. Negative control of MAP-2, which contained no reverse transcriptase, showed no PCR products.

Immunofluorescence: Breast cancer cells MCF-7 and MCF-10A were grown on Falcon culture slides (Franklin Lakes, NJ). Cells were fixed in 3 % paraformaldehyde in PBS for 15 min at room temperature and permeabilized by dipping cells for 10 seconds in 100 % methanol (-20°C). After 3 washes in PBS each for 5 minutes the cells were incubated in 1% BSA, PBS pH 7.5 for 30 min to block unspecific binding of the antibodies. The cells were incubated with primary antibody in 1% BSA, PBS pH 7.5 over night at 4 °C and washed with PBS pH 7.5, 3-times for 10 min. The cells were then incubated with secondary antibody conjugated with green fluorescent protein, in 1% BSA, PBS pH 7.5 for 60 min at room temperature followed by 3 washes with PBS pH 7.5 each for 10 min. After brief rinses with PBS the cells were mounted in anti-fade medium (Molecular probes, Eugene, OR) and observed under a fluorescent microscope.

Cell growth/cell viability by MTT assay: The effect of docetaxel on the viability of cells challenged with EGF (50-150 ng/ml) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at 2×10^5 cells/well in 200 μ l of DMEM complete medium in a 96-well microtiter plate. Twenty four hours later cells were serum starved for 24 h and then challenged with EGF. Twelve hours later cells were treated with 100 ng/ml docetaxel. Each treatment was repeated in 10 wells and the plate incubated for 24 h at 37 °C in a humidified chamber. Following 24 h of incubation, 4 μ l of MTT reagent (5 mg/ml in PBS) was added to each well and the plate incubated for 2 h. The plate was centrifuged at 1800 rpm for 5 min at 4 °C. The MTT solution was removed from the wells by aspiration and the Formazan crystals were dissolved in 150 μ l of DMSO. Absorbance was recorded on a microplate

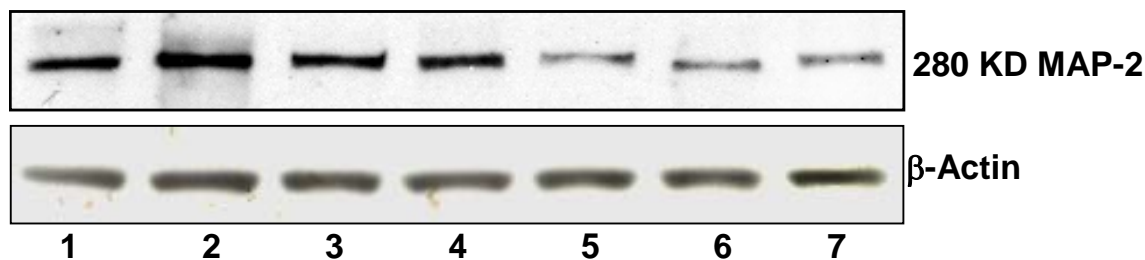
reader at 540 nm wavelength. The effect of doxorubicin on growth inhibition was assessed as percentage cell viability where vehicle-treated cells were taken as 100% viable.

PepChip kinase screening. PepChip kinase, a microarray containing 1,152 synthetic kinase substrate peptides, was purchased from Pepscan systems (Lelystad, Netherlands). Cell lysates from control and EGF (100 ng/ml) treated cells were prepared in 25 mmol/L HEPES (pH 7.6), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton X-100, 0.1 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride, and the cleared supernatants were used for the screening according to the instructions of the manufacturer. The microarrays were scanned by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with a resolution of 50 μ m. Evaluation of the data was done using ImageQuant software. Normalization of signal intensity of each peptide was done according to the instructions of the manufacturer.

RESULTS AND DISCUSSION:

Protein and mRNA expression of MAP-2 in breast cancer cells: Since these were first studies to define the role of microtubule associated protein-2 (MAP-2) in breast cancer, we first evaluated the expression of MAP-2 in a range of breast cancer cell lines. MAP-2 is primarily expressed in the neuronal cells where it stabilizes the cytoskeletal structure during assembly of the microtubules. All cells were grown till 70-80% confluent at which time they were harvested for preparation cell lysates and extraction of RNA followed by western blot analysis and RT-PCR. We observed that the expression of MAP-2 was higher in cells that over expressed the EGFR receptor. All breast cancer cells examined were found to express varying degrees of MAP-2, however when compared on the basis of their EGFR status, we observed that breast cancer cells that over expressed EGFR also had higher expression of MAP-2. This observation on the expression of MAP-2 was examined at both protein and mRNA levels with similar results (Fig. 1 A & B).

(A)



(B)

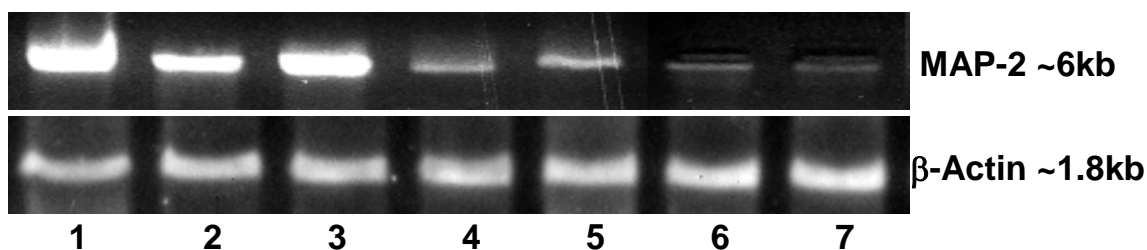
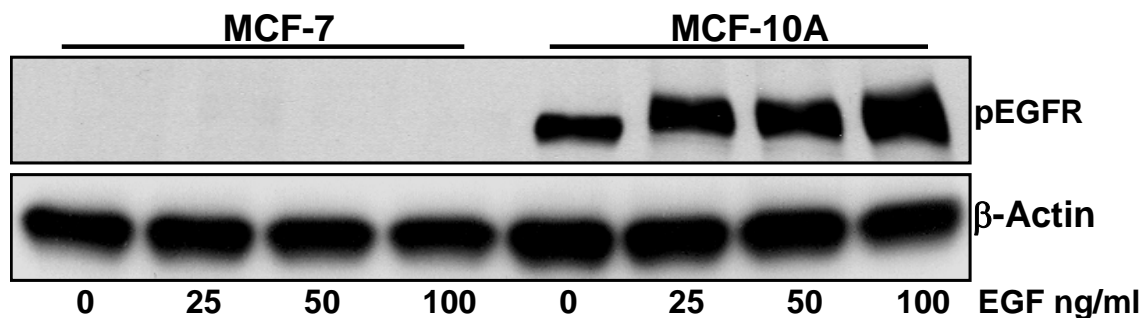


Figure 1: Protein (A) and mRNA (B) expression of MAP-2 in normal and human breast cancer cells. Lanes: (1) Normal human breast epithelial cells (2) HCC-38 (3) HCC-70 (4) MCF-7 (5) AU-565 (6) HCC-1419 (7) MCF-10A.

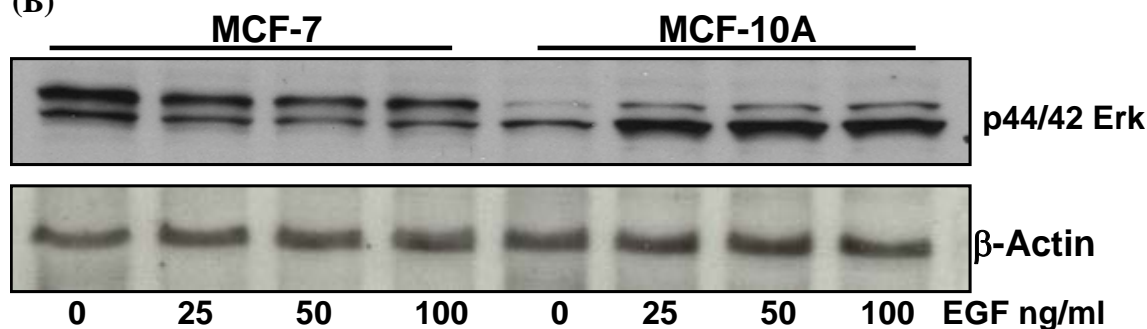
MAP-2 expression in breast cancer cells is regulated by EGFR: In order to investigate whether the expression of MAP-2 is regulated by the expression of EGFR we selected two cell lines from among the range of breast cancer cells based on their responsiveness to epidermal growth factor (EGF). Two cell lines MCF-7 and MCF-10A were serum starved for 24 h and then challenged with increasing doses of EGF (25, 50 and 100 ng/ml). Twelve hours post EGF treatment cells were harvested and examined for the expression of phosphorylated EGFR, Erk1/2 and MAP-2. Additionally, cells were grown on culture slides for examination of MAP-2 by immunofluorescence.

We observed that MCF-7 cells were weakly stimulated by EGF whereas MCF-10A was strongly stimulated when challenged by increasing doses of EGF. Responsiveness to EGF stimulation was evaluated by expression of phosphoEGFR and downstream MAPK Erk1/2. Phospho EGFR expression was not observed in MCF-7 cells challenged with EGF, however, MCF-10A cells responded with a dose-dependent increase in EGFR phosphorylation and downstream activation of Erk1/2. We next examined the expression of MAP-2 in the cell lines challenged with EGF and observed that although MAP-2 expression increased with increasing doses of EGF, its expression was almost completely lost at higher concentrations of EGF treatment (Fig. 2, A-D).

(A)



(B)



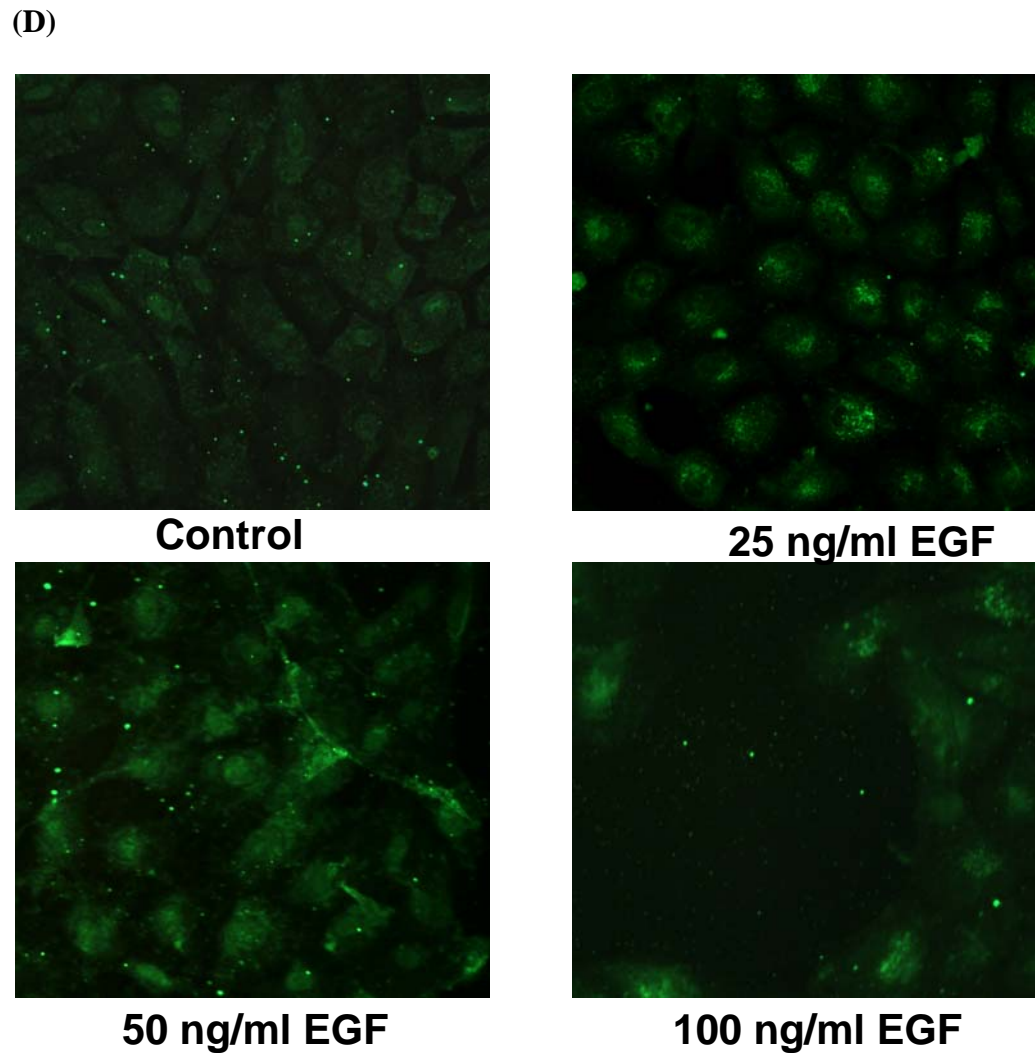
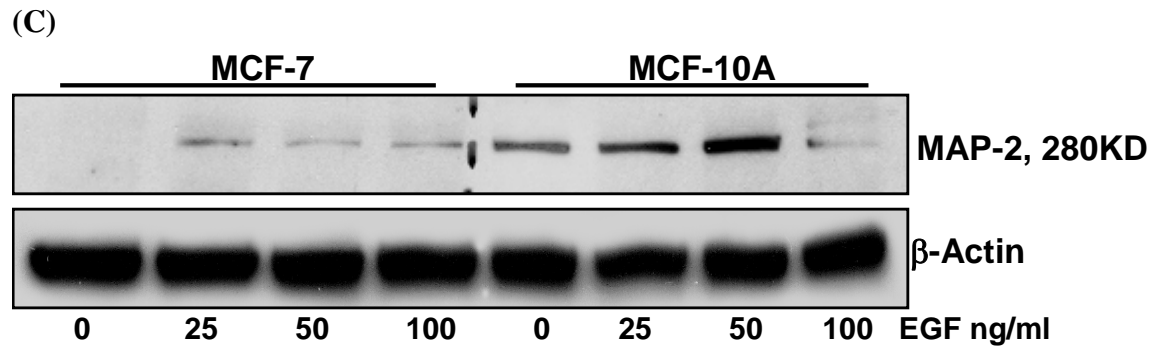


Fig. 2 (A-D). Effect of EGF stimulation on the expression of phosphor-EGFR, Erk1/2 and MAP-2 in breast cancer cells MCF-7 and MCF-10A. Cells were serum starved for 24 h and then challenged with increasing doses of EGF (25, 50 and 100 ng/ml). Twelve hours post EGF treatment cells were harvested and examined for the expression of phosphorylated EGFR, Erk1/2 and MAP-2. Additionally, cells were grown on culture slides for examination of MAP-2 by immunofluorescence (D).

MAP-2 expression is lost in EGF responsive cells during sustained activation of EGFR. The loss of MAP-2 at higher concentration was an interesting observation that could point towards a possible mechanism of resistance in breast cancer patients with EGFR over expression. In order to further confirm the loss of MAP-2 expression in breast cancer, MCF-10A cells were treated with increasing doses of EGF. This was approached in two ways. In the first approach cells MCF-10A cells were treated with increasing doses of EGF (0, 50, 75, 100, 150, 200 ng/ml) for 24 hours and cell lysates prepared for immunoblot analysis. In the second approach six groups of cells were treated with EGF (25 ng/ml) each day for five days in such a way that cumulative EGF concentration in each group was 0, 25, 50, 75, 100 and 125 ng/ml. In either approaches treatment of cells with increasing concentrations of EGF resulted in decrease in MAP-2 expression suggesting a loss of the protein. Based on these observations we suggest that with constant EGF stimulation, cells lose their MAP-2 expression that is required for microtubule stabilization and subsequent cell cycle arrest. It would be of interest to examine the expression of MAP-2 in tissues of breast cancer patients over-expressing the EGFR receptor; however this study was beyond the scope of the present investigation. The loss of MAP-2 expression would have implications in treatment of breast cancers over expressing the EGFR receptor and resistant to conventional therapy.

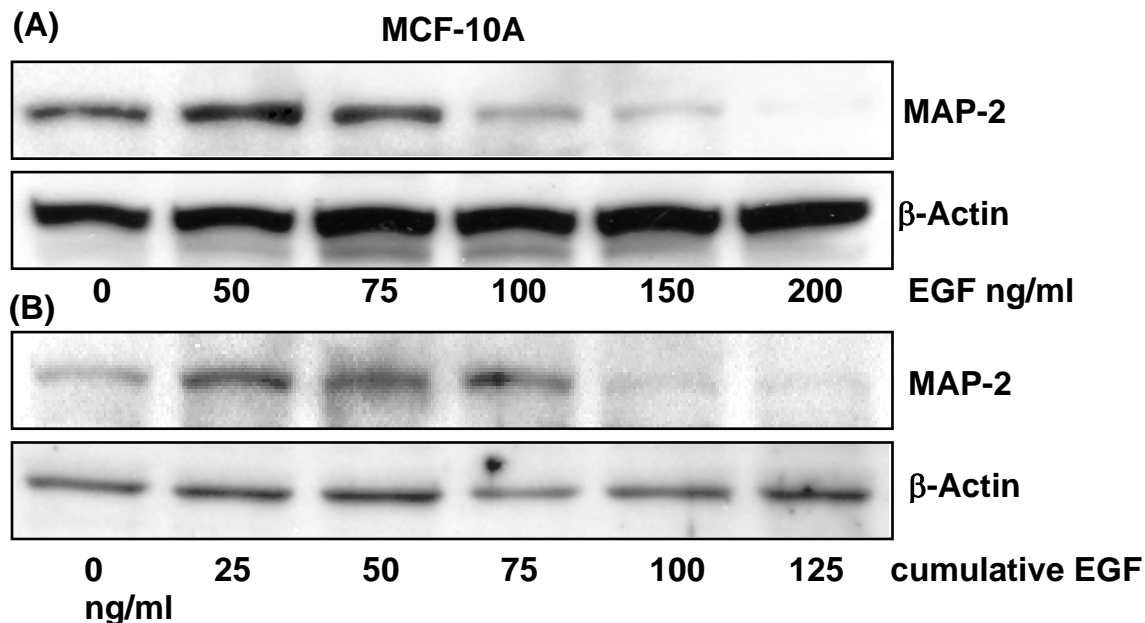


Fig. 3. (A-B). Effect of increasing doses of EGF on the expression of MAP-2 in MCF-10A. Cells were serum starved for 24 h and then challenged with (A) increasing doses of EGF (0, 50, 75, 100, 150 and 200 ng/ml) and (B) increasing cumulative doses of EGF (0, 25, 50, 75, 100 and 125 ng/ml). EGF treated cells were harvested and examined for the expression of phosphorylated MAP-2.

Loss of MAP-2 expression during sustained activation of EGFR signaling in EGF responsive cells is inhibited by blocking EGFR signaling: In order to establish whether EGFR

receptor activation is responsible for loss of MAP-2 expression in MCF-10A cells, EGFR induced signaling was inhibited by C225 an antibody against EGFR. C225 treated cells were then challenged with increasing doses of EGF (0, 25, 50, 75 and 100 ng/ml). It was observed that EGFR inhibitor C225 partially inhibited EGF induced signaling and as a consequence also prevented loss of MAP-2 expression which was observed in earlier experiments at doses >75 ng/ml. These experiments suggest that loss of MAP-2 expression proceeds through increased activation of the EGFR signaling and any reduction in this signaling prevents loss of MAP-2. These experiments also point out that a certain threshold level of EGFR signaling is required to achieve a loss of MAP-2 expression and any decrease in this threshold probably is insufficient to result in loss of MAP-2.

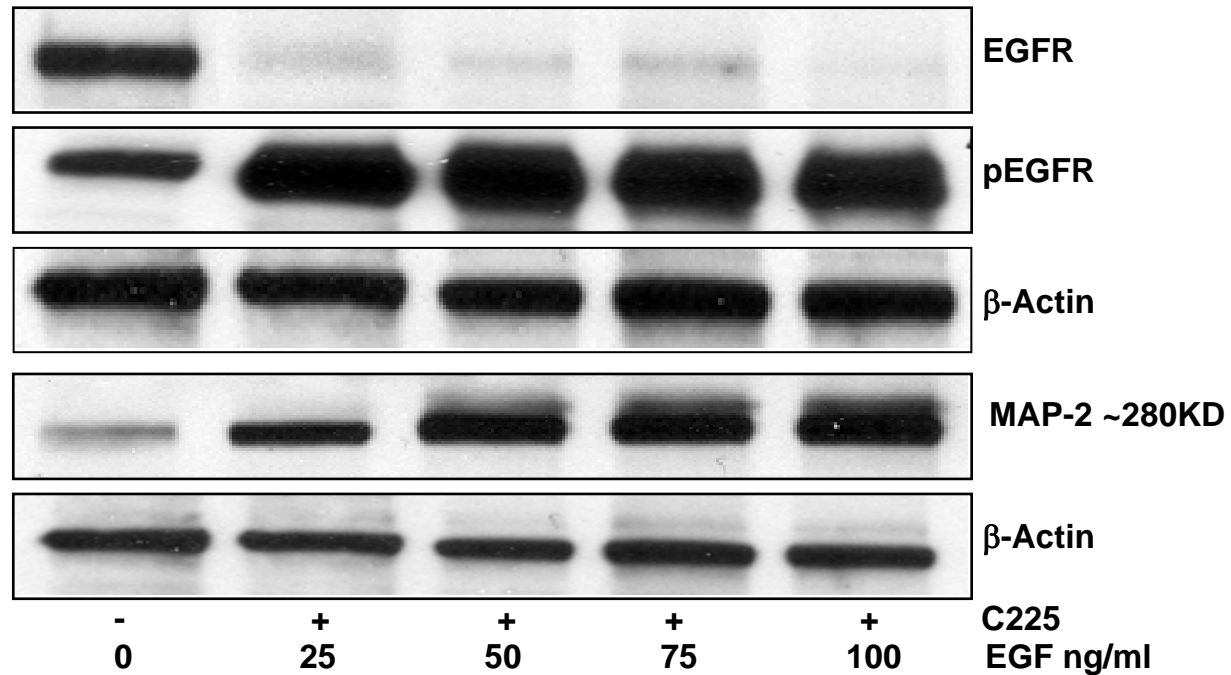


Fig. 4. Effect of increasing doses of EGF on the expression of MAP-2 in MCF-10A pretreated with C225, an antibody against EGFR. Cells were serum starved for 24 h, treated with and then challenged with (A) increasing doses of EGF (0, 50, 75, 100, 150 and 200 ng/ml) and (B) increasing cumulative doses of EGF (0, 25, 50, 75, 100 and 125 ng/ml). EGF treated cells were harvested and examined for the expression of phosphorylated EGFR and MAP-2.

Effect of Docetaxel treatment on cells challenged with EGF: In our next series of experiments we sought to understand how cells would respond to microtubule disrupting agents after they are challenged with increasing doses of EGF. MCF-10A cells were serum starved in 96-well microtiter plates, followed for EGF challenge and then treated with docetaxel. We found increasing resistance to growth inhibition by docetaxel in cells that were challenged with higher concentrations of EGFR (Fig. 3). This suggested that over expression of EGFR signaling results in loss of MAP-2 in breast cancers could and this could in fact be responsible for resistance to therapeutic agents.

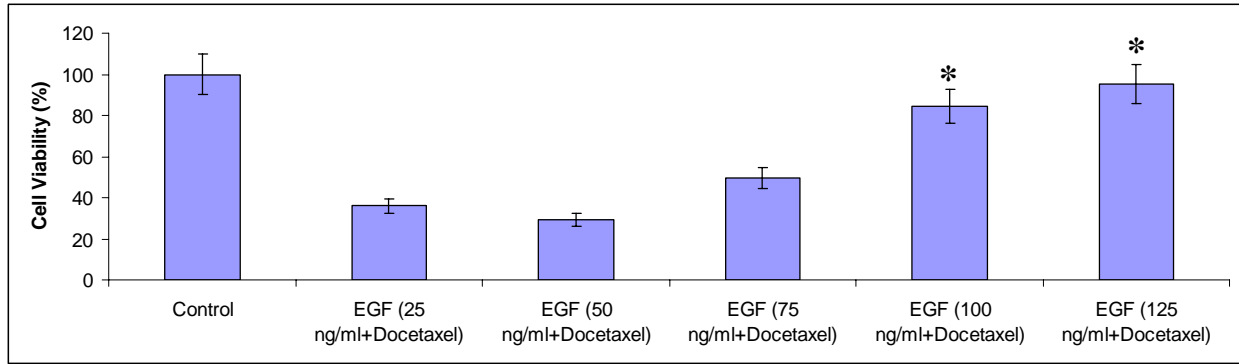


Fig. 5. Effect of docetaxel treatment on cell growth in breast cancer cells challenged with increasing doses of EGF. With increasing doses of EGF cells become refractory to docetaxel treatment. Students t-test, * $p < 0.001$ compared to control.

Effect of EGF stimulation on the status of whole cell kinases: In order to understand the underlying mechanism of loss of MAP-2 during sustained activation of the EGFR signaling, we hypothesized this increased loss may be occurring due to increased activity of kinases downstream of the EGFR. To achieve our objective we utilized a PepChip kinase array to screen the status of whole cell kinases in control and EGF (100 ng/ml) treated cells. Interestingly, we observed increased kinase levels of several substrates that are activated by EGFR signaling. In particular increased levels of protein kinases, mitogen activated protein kinases (MAPK) such as JNK and Erk were observed (Fig. 6 arrows). These observations suggest under conditions of increased kinase activity, MAP-2 is hyper phosphorylated and eventually degraded resulting in loss of MAP-2 from the system.

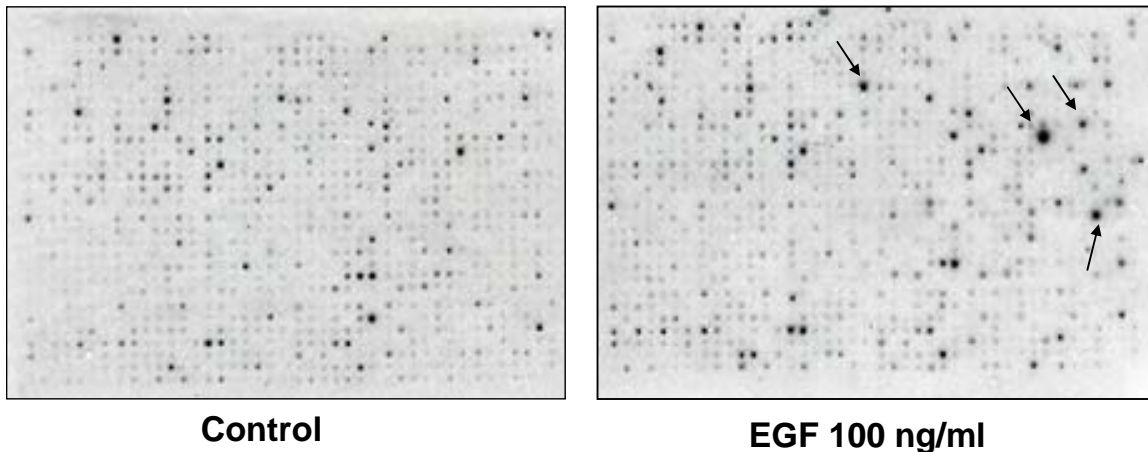


Fig. 6. Effect of EGF treatment on whole cell kinase levels in MCF-10A cells. Cells were serum starved for 24 h, treated with and then challenged with EGF (100 ng/ml) and followed by kinase array using the PepChip kinase assay and following the manufacturer's protocol. Arrows indicate kinases that exhibit increased expression compared to the corresponding control.

Significance and future studies:

Development and progression of breast cancer is associated with the accumulation of changes involving number of genes. One such change is the increased resistance to therapeutic drugs a phenomenon dreaded by the patient and the physician alike. Several factors have been found to be involved in the development of this resistance; however, no clear benefit for patients treated with 'resistance-modifier' agents has yet been demonstrated. Understanding how breast cancer cells escape this control could result in better management of the disease. This study identifies a potential role of MAP-2 expression in breast cancers over expressing the EGFR receptor. Data outlined in these studies suggest that over expression of EGFR signaling in breast cancers could, in fact, be responsible for resistance to therapeutic agents. The loss of MAP-2 expression could have implications in treatment of breast cancers over-expressing the EGFR and exhibiting resistance to conventional therapy. Results of these studies could have long term therapeutic implications against breast cancer.

REPORTABLE OUTCOMES:

ABSTRACTS

1. Adhami VM and Mukhtar, H. Association between microtubule associated protein-2 and the EGFR signaling in breast cancer. Presented at the 2005 Era of Hope meeting, June 8-11, Pennsylvania Convention Center, Philadelphia, PA, 2005.
2. Adhami VM, Zaman N, Sarfaraz S and Mukhtar H. Loss of MAP-2 during sustained activation of EGFR in breast cancer cells leads to resistance to conventional chemotherapeutic drug docetaxel. Presented at the 97th Annual meeting of the American Association for Cancer Research, April 1-5, Washington, DC, 2006.

CONCLUSIONS

This study identifies a potential role of MAP-2 expression in breast cancer cells over expressing the EGFR receptor. Data outlined in these studies suggest that over expression of EGFR signaling in breast cancers could, in fact, be responsible for resistance to therapeutic agents. As a result of sustained activation of the EGFR, MAP-2 expression is lost. The loss of MAP-2 expression occurs as a consequence of increased kinase activity following EGFR stimulating of downstream signaling. This could have implications in treatment of breast cancers over-expressing the EGFR and exhibiting resistance to conventional therapy.

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